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The insulin-like growth factors (IGF) are involved in processes leading to tumorigenesis and metastasis. The IGFs stimulate growth of mammary epithelial cells, the site of origin of ductal breast carcinomas. Their ability to stimulate growth is modulated by IGF binding protein-3. The goal of these studies is to determine how IGFBP-3 enhances IGF action. Two established cell lines genetically engineered to express IGFBP-3 serve as the experimental models. We have found that the ability of IGFBP-3 enhances IGF-I stimulated cell cycle progression in these cells and if IGF-I phosphorylates IGFBP-3. Establishing whether or not the IGF signaling cascade results in phosphorylation of IGFBP-3 is central to the overall hypothesis that intracellular IGFBP-3 plays a role in IGF-I stimulation of cell cycle progression. Further work in this area using breast tumor specimens will determine whether this pathway is disrupted in breast cancer. Potential therapies for breast cancer may include treatments that alter phosphorylation or dephoshorylation of the IGFBP-3 protein.

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INTRODUCTION:

The insulin-like growth factors (IGF) are involved in processes underlying tumorigenesis and metastasis, including cell cycle progression, inhibition of apoptosis, and cell migration. High circulating levels of IGF-I in pre-menopausal women have been shown to be associated with an increased risk of breast cancer, supporting a role for IGFs in tumor progression (1). The IGFs stimulate growth of both normal and transformed mammary epithelial cells, the site of origin of ductal breast carcinomas. Their ability to stimulate growth is modulated by IGF binding protein-3. Clinical data suggest that IGFBP-3 may promote tumor growth e.g. highly malignant breast tumors make more IGFBP-3 compared to tumors with a more positive prognosis and high serum levels of IGFBP-3 are associated with poor prognosis and a decrease in disease free survival (2). This is supported by in vitro data showing that breast tumor cells (3) and nontransformed mammary epithelial cells (4) that have been genetically engineered to constitutively express IGFBP-3 exhibit an enhanced responsiveness to IGF-I in terms of DNA synthesis. IGFBP-3 is a secreted protein and most paradigms have focused on events occurring in the extracellular environment or at the cell surface. However, we hypothesize that IGFBP-3 may function within the cell to influence IGF-I-stimulated growth. Therefore the purpose of this project is to determine if intracellular modification of IGFBP-3 by IGF-I represents an important component of IGFBP-3 action. The specific aims of this proposal are to determine if IGFBP-3 enhances IGF-I stimulated cell cycle progression in mammary epithelial cells and if phosphorylation of IGFBP-3 is required for this effect.

BODY:

The experimental models that are being used for these studies are two established cell lines that have been transfected to overexpress IGFBP-3: the estrogen receptor-positive human breast tumor cell line MCF-7 and the MAC-T non-tumorigenic bovine mammary epithelial cell line that differentiates and produces milk proteins under appropriate stimuli. To determine if cell cycle progression is enhanced in +BP3 cells in response to IGF-I, flow cytometry experiments are in progress. At this point, flow cytometry conditions have been established using wild-type MAC-T cells. Exponentially growing cells treated with or without IGF-I were collected at multiple time points. Cellular DNA was labeled with bromodeoxyuridine prior to fixation. Our next step is to determine the proportion of cells in each phase of the cell cycle at a given time for both MAC-T and MCF-7 cells transfected with IGFBP-3 or vector alone. It is anticipated that +BP3 cells treated with IGF will have a greater proportion of cells in G2/M and S phase and a smaller number in G0/G1 compared to mock cells treated with IGF by the later time points. In addition, since our previous results (4) have shown that MAC-T +BP3 cells have a very low basal rate of DNA synthesis relative to mock cells in the absence of IGF, we also anticipate that +BP3 cells should have a greater number of cells in G0/G1 compared to mock cells in the absence of IGF.

Since we have already shown that cell proliferation (both ^{3}H-thymidine incorporation assays to measure DNA synthesis as well as direct determination of cell numbers) is enhanced in both cell types overexpressing IGFBP-3, a difference in cell cycle progression is anticipated. Therefore we have expanded this aim to determine the mechanism that accounts for the increased responsiveness of IGFBP-3 expressing cells to IGF-I. Since we found no difference in the affinity or number of IGF-I receptors on the cell surface, we speculated that downstream effector molecules may be enhanced. This was proven true in our nontransformed immortalized bovine mammary epithelial cell line i.e. the phosphatidyl inositol 3-kinase (PI3K) pathway was

augmented in +BP3 cell. Phosphorylation of Akt was stimulated to a greater degree by IGF-I, as well as for an extended time period compared to mock transfected control cells (mock). In addition, +BP3 cells also exhibited enhanced activation of Akt in response to TGF-α. The later response suggests that IGFBP-3 acts downstream of the IGF receptor itself. The details of these studies are reported in reference (5). Recently it has been reported that exogenous IGFBP-3 enhances the ability of EGF to activate signaling cascades in the nontumorigenic human mammary epithelial cell line, MCF-10A (6), supporting our findings. We have now found similar results with the MCF-7 +BP-3 breast tumor cells. In response to IGF-I, Akt activation is enhanced. Since it has been reported that addition of exogenous IGFBP-3 to MCF-7 cells inhibits the ability of IGF-I to activate signal transduction cascades, this is further evidence supporting the idea that IGFBP-3 may modulate intracellular signaling through both extracellular and intracellular mechanisms (7).

The ability of IGF-I to phosphorylate IGFBP-3 will be determined by metabolic labeling with ^{32}P-orthophosphate. Cell lysates will be collected at time points over the course of 1 h and immunoprecipitated with IGFBP-3 antisera. Immunoprecipitates will be analyzed by electrophoresis, dried, autoradiographed, and quantitated by phosphorimager analysis, or immunoblotted for determination of total IGFBP-3. In addition to this approach, we have begun studies using standard immunoprecipitation approaches to determine if IGFBP-3 coprecipitates with proteins that are phosphorylated on either tyrosine or serine/threonine. Since DNA dependent-protein kinase, itself a serine-threonine kinase, has very recently been shown to phosphorylate IGFBP-3 in vitro (8), we are very interested in pursuing approaches that utilize the later antibodies. In addition, if we have problems with the metabolic labeling approach we are considering proteomic approaches using 2-dimensional gel analysis.

It should be noted that the postdoctoral fellow (Dr. Grill) working on this project left Dr. Cohick's laboratory to accept a full-time position when the cell cycle progression work was ongoing during the first year of this project and a one-year no-cost extension was approved. After a significant time lag, a new postdoctoral candidate was identified. However, it took six months for her Visa to be approved, and she just recently joined the lab in March. As a result, a second one-year extension has been approved for this project. It is anticipated that in the coming year the cell cycle progression studies and IGFBP-3 phosphorylation studies will be completed.

KEY RESEARCH ACCOMPLISHMENTS:

- A significant finding to date is that both nontransformed mammary epithelial cells as well as breast tumor cells engineered to express IGFBP-3 exhibit enhanced signaling through the PI3Kinase pathway in response to IGF-I.

REPORTABLE OUTCOMES:

- Grill CJ, Sivaprasad U, Cohick WS. 2002 Constitutive expression of IGF binding protein-3 by mammary epithelial cells alters signaling through akt and p70S6 kinase. J Mol Endocrinol 29:153-162 (a portion of Dr. Grill's salary was supported through this grant).

- Grill CJ, Cohick WS. 2001 Potentiation of IGF-I action in mammary epithelial cells expressing IGFBP-3 involves alterations in the PI3 kinase signaling cascade. 83rd Annual Meeting of the Endocrine Society, p 189 (a portion of Dr. Grill's salary was supported

through this grant).

- The postdoctoral training offered to Dr. Constance Grill through support from this grant contributed to her success in obtaining employment by Schering-Plough in the Division of Oncology.

- This award helped support the Ph.D. training of Usha Sivaprasad, who will be awarded her Ph.D. degree in December, 2003.

- It is anticipated that the remaining work supported by this grant will result in one additional abstract to be presented at a national meeting and at least one additional publication.

- It is anticipated that the remaining work supported by this grant will form the basis of a grant to the American Cancer Society in October 2003 as well as an Idea grant to be submitted to the DOD Breast Cancer Research Program in May, 2004.

CONCLUSIONS:

Establishing whether or not the IGF signaling cascade results in phosphorylation of IGFBP-3 is central to the overall novel hypothesis that intracellular IGFBP-3 plays a role in IGF-I stimulation of cell cycle progression. Future studies will determine the actual phosphorylation sites involved, including mutational analysis and generation of stable cell lines expressing mutated IGFBP-3 for use in functional studies. In addition, studies will be designed to determine the phase(s) of the cell cycle that is influenced by IGFBP-3. Further work in this area using breast tumor specimens will determine whether this pathway is disrupted in breast cancer. In addition this work will help to explain why IGFBP-3 has been reported to have opposing effects on mammary cell growth. Potential therapies for breast cancer may include treatments that alter phosphorylation or dephosphorylation of the IGFBP-3 protein.

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